Antisera against a Guanine Nucleotide Binding Protein from Retina Cross-React with the $\beta $ Subunit of the Adenylyl Cyclase-Associated Guanine Nucleotide Binding Proteins, N$_{\text{s}}$ and N$_{\text{i}}$

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Antisera against a guanine nucleotide binding protein from retina cross-react with the β subunit of the adenylyl cyclase-associated guanine nucleotide binding proteins, N_s and N_i

(transducin/cAMP/receptor coupling/transmembrane signaling)

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ABSTRACT **Antisera were produced in rabbits against a guanine nucleotide binding protein (N protein), transducin, purified from bovine retina. Antiserum AS/1, which recognized all three subunits (α, β, and γ) of the holoprotein, was tested for cross-reactivity with the subunits of the adenylyl cyclase [adenylate cyclase; ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]-associated stimulatory (N_s) and inhibitory (N_i) N proteins purified from human erythrocytes. AS/1 showed strong reactivity against the β subunits of both N_s and N_i but failed to cross-react with either the α or γ subunits of N_s and N_i. Seven additional antisera against transducin reacted with the β subunits but not with the α or γ subunits of N_s and N_i. A single antiserum against transducin reacted with the α subunit of N_i but not of N_s. Immunostaining of the β subunits of N_s and N_i was proportional to the amount of β subunit blotted and to the antiserum concentration. Immunostaining of either human erythrocyte or bovine cerebral cortical plasma membrane proteins with AS/1 showed a single band, comigrating with the β subunit of transducin; this band was absent in bovine erythrocyte membranes. Estimation of the amount of β subunit by immunoblotting with AS/1 showed that the β subunit comprises ≈2% of bovine cerebral cortical plasma membrane protein, ≈100-fold more than in human erythrocyte membranes. These findings provide immunochemical evidence for similarities in the β subunits and differences in the α and γ subunits of this family of N proteins. Antisera against transducin react specifically with the β subunits of N_s and N_i in crude plasma membranes and, thus, can serve as specific probes for the β subunit.**

Retinal-rod outer-segment (ROS) disc membranes contain a guanine nucleotide binding protein (N protein), also termed transducin (TD) (1), that couples light activation of rhodopsin to increased cGMP phosphodiesterase activity (2). TD shows functional (1, 2) and structural (3, 4) homology to the stimulatory and inhibitory N proteins (N_s and N_i, respectively) associated with adenylyl cyclase [adenylate cyclase; ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. N_s and N_i are intrinsic membrane proteins, present in low abundance in plasma membranes (5). Purification of substantial amounts of either protein has not been feasible with available methods, nor have antisera been readily produced (5). TD, in contrast, comprises as much as 4% of ROS membrane protein (6) and is readily purified in aqueous buffers (6). Therefore, we produced antisera in rabbits against purified bovine TD and tested the cross-reactivity of these antisera against N_s and N_i. We now report that: (*i*) most (eight of nine) antisera against TD cross-react with the β subunits but not with the α or γ subunits of purified N_s and N_i; (*ii*) a single antiserum against TD cross-reacts with the α subunit of N_i but not of N_s; and (*iii*) antisera against TD can be used as specific probes for the β subunits of N_s and N_i in crude plasma membranes.

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EXPERIMENTAL PROCEDURES

Materials. Bovine blood, cerebral cortex, and retinas were obtained from a local slaughterhouse. Human erythrocytes were either freshly drawn or obtained from outdated bank blood. Materials for gel electrophoresis and immunoblotting were obtained from Bio-Rad with the exception of peroxidase-conjugated goat anti-rabbit Ig (Kierkegaard & Perry) and radioiodinated staphylococcal protein A (New England Nuclear). The sources of other materials have been cited (4).

Protein Purification. Retinas were dissected from freshly obtained bovine eyes, and ROS membranes were prepared (7). TD was purified from ROS membranes essentially as described (6) except that both isotonic and hypotonic elutions were performed six instead of three times. TD was eluted with hypotonic buffer containing 40 μM guanylylimidodiphosphate. Purification of N_s, N_i, and the M_r 40,000 protein (which contains the M_r 35,000 and M_r 5,000 subunits of N_s and N_i) was as described (8). For some experiments (see Fig. 2) a fraction containing both N_s and N_i (after the third DEAE-Sephacel step and before hydroxylapatite chromatography) was used.

Immunization with TD. Six New Zealand White rabbits (AS/1–AS/6) were injected intradermally with 100 μg of purified TD per animal in complete Freund's adjuvant, followed by an injection of 50 μg in incomplete Freund's adjuvant 2 wk later. Some animals received additional booster injections subsequently. Six other animals (CW/1–CW/6) were injected in an identical way with purified TD treated with 6 M urea and 10% mercaptoethanol before emulsion in either complete or incomplete Freund's adjuvant. Animals were bled before and at intervals after immunization, and heat-inactivated sera were collected.

Membrane Preparations. Human and bovine erythrocyte membranes were prepared by hypotonic lysis as described (9). Bovine cerebral cortex was homogenized with a Teflon pestle in buffer (10 mM Tris·HCl, pH 7.5/2 mM $MgCl_2$/0.25 M sucrose) and was centrifuged at low speed to remove unbroken cells and then at 10,000 × *g* for 10 min to collect a crude membrane pellet. This was washed twice with buffer, resuspended in buffer, frozen, and stored in liquid nitrogen.

Electrophoresis. $NaDodSO_4$/polacrylamide gel electrophoresis (10) and discontinuous $NaDodSO_4$ urea/polyacry-

Abbreviations: N proteins, guanine nucleotide binding proteins; N_s, stimulatory N associated with adenylyl cyclase; N_i, inhibitory N associated with adenylyl cyclase; TD, transducin, the retinal N protein; ROS, rod outer segments.

lamide gradient gel electrophoresis (4) were performed as described. Purified proteins were prepared for electrophoresis as described (4), and membranes were prepared for electrophoresis by suspending in sample buffer (10) and boiling for 5 min.

Blotting and Immunostaining. Proteins were transferred from gel to nitrocellulose paper with constant current (150 mA) for 12 hr in a Bio-Rad transblot apparatus (11). After transfer, the paper was incubated in 10 mM Tris·HCl, pH 7.5/500 mM NaCl with 3% gelatin to block nonspecific protein binding. The paper was then incubated for 4–24 hr at room temperature in the same buffer containing 1% gelatin and various dilutions of rabbit antisera. After being washed, the paper was incubated with a second antibody (1 μg of peroxidase-conjugated goat anti-rabbit IgG per ml) for 2 hr at room temperature. After repeated washings, papers were stained in 8.3 mM Tris·HCl, pH 7.5/415 mM NaCl/20% (vol/vol) methanol/0.015% H_2O_2/0.5 mg of 4-chloro-1-naphthol per ml for 10 min at room temperature. Alternatively, after incubation with the first antibody, papers were washed and then incubated with radioiodinated protein A (about 100,000 cpm/ml) in 50 ml of Tris-buffered saline containing 1% gelatin for 45 min at room temperature. The paper was then washed extensively in Tris-buffered saline containing 0.05% Tween 20 and dried. Autoradiography was then performed with Kodak XAR film with DuPont Cronex image-intensifying screens at −70°C. Autoradiograms were used to identify the relevant band on nitrocellulose blots, and then these were cut out for assay in a gamma spectrometer to quantitate immunoreactive protein. Protein concentration was determined as described (12).

RESULTS

We produced antisera in rabbits against TD purified from bovine retina. TD is a heterotrimer consisting of α, β, and γ subunits (1). Therefore, we tested each antiserum for reactivity against the subunits of TD separated by $NaDodSO_4$/polyacrylamide gel electrophoresis. Although each animal was injected with holoprotein, the antisera varied in their reactivity with the subunits. Antiserum AS/1 showed the strongest reactivity against all three subunits.

Since TD, N_s, and N_i are functionally homologous (2, 5) and show structural similarities as determined by peptide mapping (3), we tested the antisera against TD for cross-reactivity with the subunits of purified N_s and N_i. First, N_s, N_i, the M_r 40,000 protein, and TD were subjected to discontinuous $NaDodSO_4$/urea/polyacrylamide gradient gel electrophoresis to identify their subunit composition (Fig. 1*A*). In parallel, the proteins were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, and the separated proteins were transferred to nitrocellulose paper. The paper was then immunostained by using antiserum AS/1 (Fig. 1*B*). AS/1 showed strong reactivity against all three TD subunits. AS/1 showed strong cross-reactivity with the β subunits of N_s, N_i, and the M_r 40,000 protein, but there was no specific reaction with either the α subunits of N_s and N_i or the γ subunits of N_s, N_i, and the M_r 40,000 protein (Fig. 1*B*). No staining was observed with preimmune serum from rabbit AS/1 (not shown). In addition to AS/1, eight other antisera against bovine TD were tested for cross-reactivity with the subunits of N_s and N_i. For these experiments, immunoblotting of TD and of a mixture of N_s/N_i was performed (Fig. 2). The nine anti-TD antisera all reacted to various degrees with all three subunits of TD; nonimmune serum showed no reactivity. Eight of nine anti-TD antisera recognized only the β subunits of N_s and N_i. A single antiserum, CW/6, cross-reacted with the α subunit of N_i but not of N_s (Fig. 2).

We next examined the specificity of TD antisera in immunostaining relatively crude plasma membrane protein

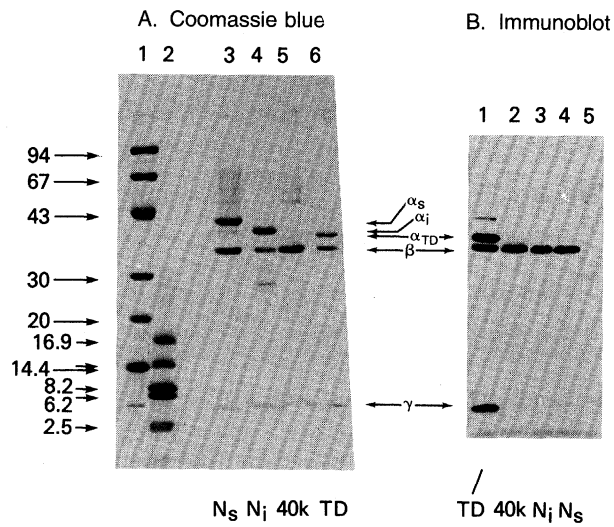

FIG. 1. Discontinuous $NaDodSO_4$ urea/polyacrylamide gradient gel electrophoresis of N_s, N_i, the M_r 40,000 protein, and TD. Approximately 10 μg of each preparation were precipitated and subjected to discontinuous $NaDodSO_4$ urea/polyacrylamide gradient gel electrophoresis as described (4). The gel was stained with Coomassie blue. Standards (M_r shown $\times 10^{-3}$) are Pharmacia low molecular weight standards (lane 1) and cyanogen bromide fragments of myoglobin (lane 2). Protein staining shows a β subunit of M_r 35,000 and a γ subunit of $M_r \approx 5000$ in each preparation (lanes 3–6). The molecular weights of the α subunits are 42,000 for N_s (lane 3), 40,000 for N_i (lane 4), and 39,000 for TD (lane 6). The M_r 40,000 protein (40 k) lacks an α subunit. (*B*) Immunostaining of proteins in *A* blotted onto nitrocellulose paper. Proteins were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (12.5% gel) and then blotted onto nitrocellulose paper and immunostained. A 1:50 dilution of AS/1 was used and incubation with the first antibody was for 24 hr at room temperature. AS/1 reacted strongly with all three subunits of TD (lane 1) but only with the β subunits of N_s, N_i, and the M_r 40,000 protein (lanes 2–4, respectively). The faint bands (e.g., the blotted molecular weight standards in lane 5) represent nonspecific staining. The bands at the bottom of *B* represent the tracking dye, pyronin Y.

preparations. Antiserum AS/1 revealed a single specific band in immunoblots of human erythrocyte and bovine cerebral cortical plasma membranes (Fig. 3). The stained band comigrated with the β subunit of purified bovine TD. This band was not seen in bovine erythrocyte plasma membranes.

To determine if immunoblotting could be used to estimate the amount of β subunit, we subjected different concentrations of purified β subunit of TD and of N_s and N_i, as well as increasing concentrations of bovine cerebral cortical membranes, to $NaDodSO_4$/polyacrylamide gel electrophoresis, blotted these onto nitrocellulose paper, and incubated blots with TD antiserum and radioiodinated protein A. Autoradiography of the nitrocellulose blots showed that band density was proportional to the amount of β subunit loaded onto the gel. Immunoreactivity of the β subunits of TD, N_s, and N_i was approximately the same. The slightly greater immunoreactivity of the beta subunits of N_s and N_i apparent in Fig. 4 is probably due to differences in the protein measurements for the purified TD vs. the N_s and N_i preparations.

To measure the amount of β subunit in plasma membranes, we performed immunoblotting with AS/1 and radioiodinated protein A and then cut out the portion of the blot containing the β band (localized by autoradiography) to determine bound radioactivity. The amount of radioiodinated protein A bound to the β band was proportional to the amount of TD loaded onto the gel and also was a function of antibody concentration (Fig. 5*A*). A linear relationship between bound radioactivity and membrane protein also was observed for bovine cerebral cortical (Fig. 5*B*) and human

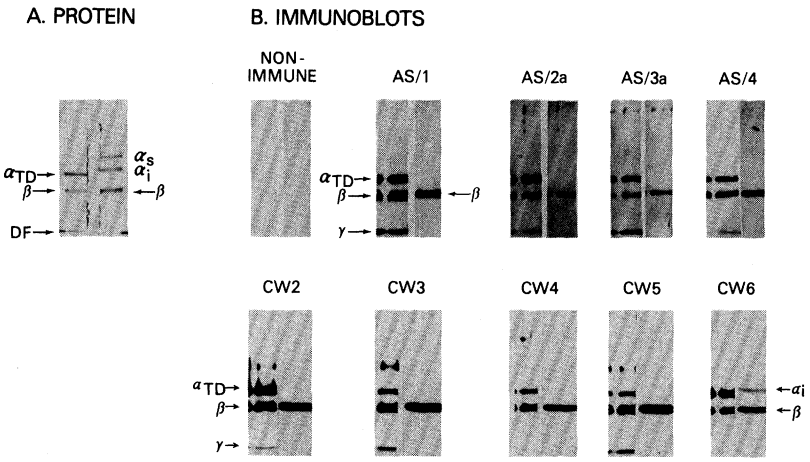

FIG. 2. (*A*) NaDodSO$_4$/polyacrylamide gel electrophoresis of TD and a mixture of N_s and N_i. Approximately 5 μg per lane of TD and of a preparation containing N_s and N_i were loaded onto a 10% polyacrylamide gel and electrophoresis was performed. The two lanes were stained with Coomassie blue and show the α and β subunits of TD (left lane) and the α subunits of N_s and N_i as well as their common β subunits (right lane). The γ subunits run at the dye front (DF) on this gel. (*B*) Immunostaining of TD and N_s/N_i. The remaining portion of the gel was blotted onto nitrocellulose paper and strips containing either TD (the left lane of each pair) or N_s/N_i (right lane of each pair) were cut and incubated with a 1:100 dilution of either normal (nonimmune) rabbit serum or one of nine different rabbit antisera raised against bovine TD (AS/1–AS/4, CW/2–CW/6) for 24 hr at room temperature. Immunostaining was then performed with peroxidase-conjugated goat anti-rabbit Ig serum as described. Each of the TD antisera reacted to various degrees with all three TD subunits, but, except for CW6, all cross-reacted only with the common β subunits of N_s and N_i. CW6 cross-reacted with the α subunit of N_i but not of N_s.

erythrocyte (Fig. 5*C*) plasma membranes. By relating the radioactivity bound to the β subunit in plasma membranes to that bound to known amounts of TD, we could estimate the amount of β subunit in plasma membranes. We estimate on this basis that the β subunit comprises between 1% and 4% of bovine cerebral cortical plasma membrane protein and ≈0.02% of human erythrocyte membrane protein. No β subunit could be detected in bovine erythrocyte membranes even at the highest concentration (300 μg) tested.

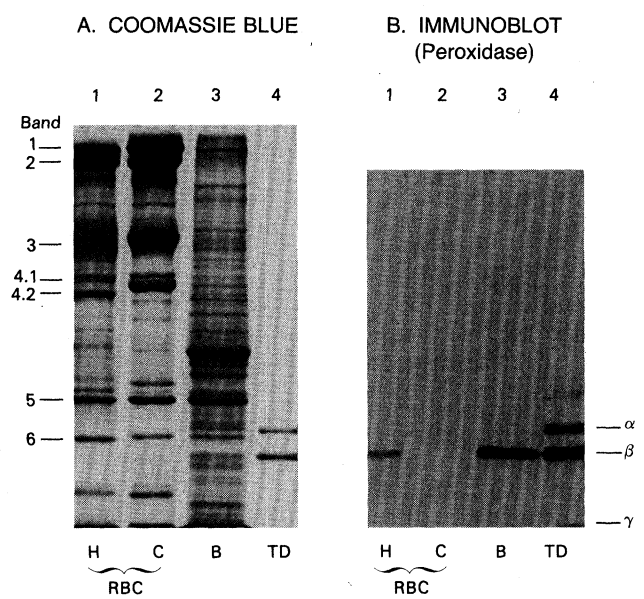

FIG. 3. (*A*) NaDodSO$_4$/polyacrylamide gel electrophoresis of plasma membranes from human erythrocytes, 200 μg (lane 1); from bovine erythrocytes, 200 μg (lane 2); from bovine cerebral cortex, 50 μg (lane 3); and from purified bovine TD, 5 μg (lane 4). The proteins in *A* were stained with Coomassie blue following electrophoresis on a 10% polyacrylamide gel. The positions of the major protein bands in human erythrocyte plamsa membranes are indicated on the left. (*B*) Immunoblot of proteins from *A* following transfer to nitrocellulose paper. The nitrocellulose paper was incubated with 1:100 TD antiserum AS/1 and stained with peroxidase-conjugated goat anti-rabbit Ig. The antiserum reacts specifically with a M_r 35,000 protein comigrating with the TD β subunit in both bovine cerebral cortical and human erythrocyte membranes. The band is not seen in bovine erythrocyte membranes.

DISCUSSION

TD, N_s, and N_i are functionally homologous in that they are N proteins that couple membrane-bound receptors (for hormones and neurotransmitters in the case of N_s and N_i and for light in the case of TD) to effector enzymes (1, 2, 5). The β subunits of TD, N_s, and N_i have been found to be virtually identical by peptide mapping of proteolytic fragments (3). The ability of TD antisera reactive with the β subunit of TD to recognize the β subunits of N_s and N_i provides immunochemical evidence for similarity in structure of the β subunits. The α subunits of TD, N_s, and N_i also have been compared by peptide mapping (3). In contrast to the findings with their β subunits, the α subunits of TD and N_i showed some similarity, with the α subunit of N_s being clearly different. We were surprised, therefore, that eight different antisera showing strong reactivity with the α subunit of TD failed

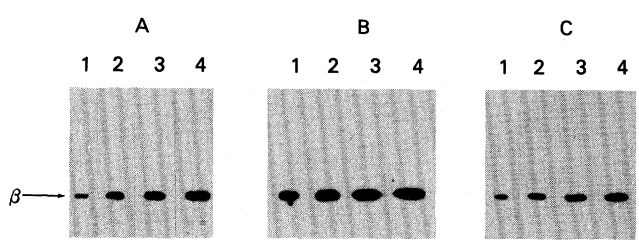

FIG. 4. Immunoreactivity of the β subunit of TD, N_s, and N_i as a function of increasing protein concentration. Proteins were loaded onto 10% polyacrylamide gels, and NaDodSO$_4$/polyacrylamide gel electrophoresis, blotting onto nitrocellulose, immunostaining using radioiodinated protein A, and autoradiography were performed as described. (*A*) The purified β–γ subunit of TD. Lanes: 1, 250 ng; 2, 500 ng; 3, 750 ng; 4, 1 μg. (*B*) The purified β–γ subunit of N_s and N_i (M_r 40,000 protein). Lanes: 1, 250 ng; 2, 500 ng; 3, 750 ng; 4, 1 μg. (*C*) Bovine cerebral cortical plasma membranes. Lanes: 1, 12.5 μg; 2, 25 μg; 3, 37.5 μg; 4, 50 μg. The arrow indicates the immunoreactive β subunit. For TD in *A*, reactivity of the γ subunit is also evident on the autoradiogram below the darker β band.

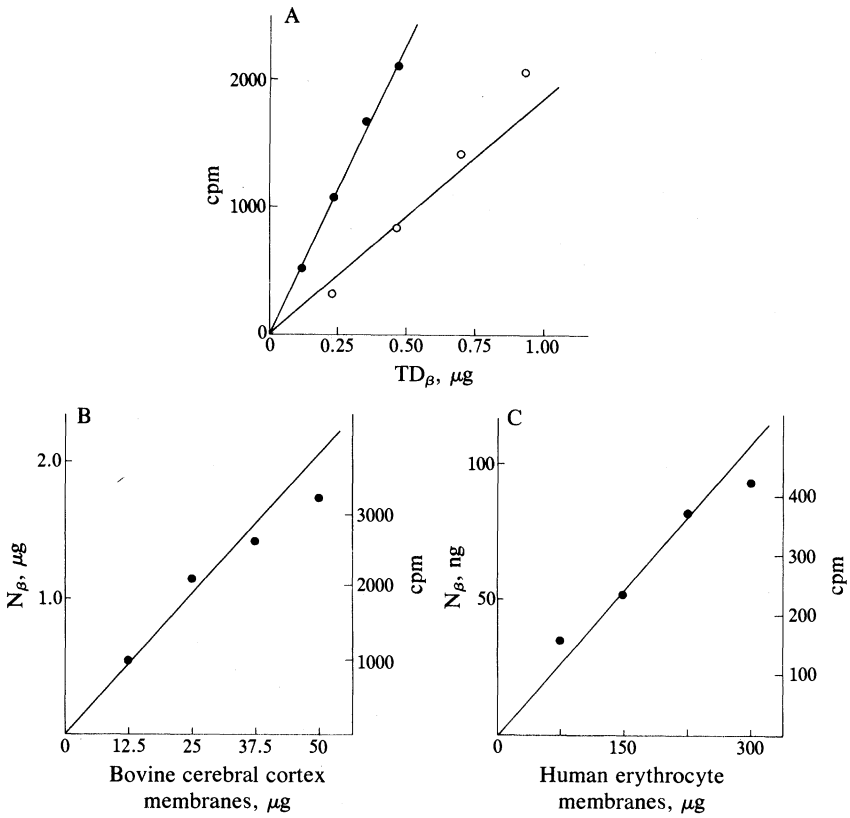

FIG. 5. Estimation of the β subunit of N_s and N_i in plasma membranes by immunoblotting. Increasing amounts of the purified β–γ subunit of TD (*A*), of bovine cerebral cortical membranes (*B*), and of human erythrocyte membranes (*C*) were loaded onto 10% polyacrylamide gels, and NaDodSO$_4$/polyacrylamide gel electrophoresis, blotting onto nitrocellulose, immunostaining with radioiodinated protein A, and quantitation of the radioactivity associated with the β subunit were performed as described. First antibody was diluted 1:50 (●) and 1:100 (○) in *A*, 1:100 in *B*, and 1:50 in *C*. Radioactivity associated with the β subunit of TD is proportional to the amount of TD loaded onto the gel and is also a function of the antibody concentration. The amount of β subunit in plasma membranes (*B* and *C*) was estimated by relating the radioactivity measurements in *B* and *C* to those found for known amounts of TD β subunit run at equivalent first antibody concentrations. Note the differences in the ordinate scales in *B* and *C*.

to cross-react with the α subunit of either N_i or N_s, even under conditions favoring cross-reactivity [high antiserum concentration, 1:50 dilution, and long (24 hr) incubation time]. A single anti-TD antiserum cross-reacted with the α subunit of N_i. It is possible that most antisera that were produced against bovine TD failed to cross-react with N_s or N_i α subunits from human erythrocytes because of species differences. This seems unlikely for several reasons: (*i*) it was clearly not the case for the β subunits; (*ii*) peptide map similarities between the α subunits of TD and N_i have been demonstrated in different species—bovine and rabbit, respectively (3); and (*iii*) perhaps most significant is that TD antisera (with the exception of CW$_6$; unpublished observations) revealed only the β subunit in bovine cerebral cortical membranes. Given the intensity of β staining in this tissue, cross-reactivity with the α subunit of N_i or N_s should have been detectable even if the concentration of α subunit were 1:10 to 1:20 of the β subunit. Instead, despite evident similarities among the α subunits, including peptide maps (3) and sites for guanine nucleotide binding and bacterial toxin modification (5), it would appear that there are sufficient differences such that we failed to detect cross-reactivity with most antisera raised against bovine TD. The α subunits of N_s and N_i clearly differ in their specificity for effector enzyme interaction (5). The TD α subunit interacts with cGMP phosphodiesterase rather than adenylyl cyclase; its ability to interact with the catalytic unit of adenylyl cyclase has not been rigorously tested. These differences in effector specificity among the α subunits may be reflected in the immunochemical differences we have found. It remains possible that antisera raised against modified forms of bovine TD could show cross-reactivity against the N_i α subunit or, less likely, against the N_s α subunit.

Although TD has long been known to contain a low molecular weight γ subunit (1), evidence for subunits of identical molecular weight in N_s and N_i has only recently been reported (4). Activation of N proteins (including N_s, N_i, and TD) by guanine nucleotides leads to a subunit dissociation reaction with the formation of free α subunits (5, 6) and β–γ complexes (4). β–γ complexes of M_r 40,000 can be isolated as a side product of the purification of N_s and N_i (8); similarly, a pure β–γ complex can be isolated from guanine nucleotide-activated TD (1, 6). It was of interest, therefore, that while anti-TD anti-sera cross-react with the β subunits of N_s and N_i, no such cross-reactivity was observed for the γ subunits. This suggests that there are important differences between the TD γ subunit and the γ subunits of the adenylyl cyclase-associated N proteins. An important question, not addressed by this study, is whether the γ subunits of N_s and N_i differ. It is possible that differences between the TD γ subunit and the γ subunits of N_s and N_i reflect differences in membrane attachment of these proteins—i.e., TD is released from membranes in aqueous buffers whereas N_s and N_i are not. It is equally possible, however, that differences in the γ subunits confer relative specificity in another interaction of this family of proteins—e.g., the interaction with different classes of receptors (stimulatory for N_s, inhibitory for N_i, and rhodopsin for TD) or the interaction of the β–γ complexes with their respective α subunits.

In addition to the structural information derived from immunochemical comparisons of TD, N_s, and N_i, our data suggest that TD antisera can be used as specific probes for the β subunits of the adenylyl cyclase-associated N proteins. Because of the difficulties in purifying substantial quantities of N_s and N_i, it has been hard to produce antisera against these proteins (5). Radiolabeling of the α subunits of N_s and N_i with cholera and pertussis toxin, respectively, has provided specific probes for studies of these subunits, but no such probe has been available for the β subunits. TD antisera provide a novel tool for this purpose. Using anti-TD antiserum AS/1, we showed that a single protein band, comigrating with TD β subunit, was specifically labeled in immunoblots of human erythrocyte and bovine cerebral cortical membranes. Although not absolutely quantitative because of potential losses during electrotransfer and washing to reduce nonspecific staining, immunoblotting with AS/1 could be

used to estimate relative amounts of β subunits in membrane preparations. The concentration of β subunits in bovine cerebral cortical membranes was found to be about 100-fold higher than in human erythrocyte membranes. This is not surprising since the mature mammalian erythrocyte is relatively deficient both in adenylate cyclase activity and in receptors coupled to N proteins. Indeed, we found that bovine erythrocyte membranes did not contain detectable amounts of β subunit. This is consistent with other findings: (*i*) no detectable N_s activity as assayed by reconstitution of membrane extracts into N_s-deficient CYC^- plasma membranes; (*ii*) no detectable radiolabeling of N_i α subunit with pertussis toxin and NAD (unpublished observations); this suggests that bovine erythrocyte membranes have lost essentially all N proteins.

Immunoblotting of the β subunits with anti-TD antisera should provide a method for studying tissue-specific differences in the amount of N proteins. Additional potential uses of such antisera include immunoaffinity purification of the β subunits of N_s and N_i (and possibly of the holoproteins) and screening of expression vector cDNA libraries for cDNA for the TD subunits as well as for the β subunits of N_s and N_i.

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